A NEW FUSION PROTEIN AND ITS USE IN AN IMMUNOASSAY FOR THE SIMULTANEOUS DETECTION OF AUTOANTIBODIES RELATED TO INSULIN-DEPENDENT DIABETES MELLITUS

FIELD OF THE INVENTION

This invention relates to a new fusion protein, its cDNA, and a vector and a cell comprising said cDNA. Furthermore, this invention relates to the use of said fusion protein in an immunoassay for simultaneous detection of autoantibodies related to insulin dependent diabetes mellitus.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

GAD65, IA2 and insulin are pancreatic proteins produced by the beta cells (for review see Atkinson and Maclaren 1993). Autoantibodies to these proteins are detected in patients with insulin-dependent diabetes mellitus (IDDM) and healthy individuals at risk for developing the disease. More than 80 % of newly-diagnosed IDDM patients have antibodies against at least one of these proteins (Baekkeskov et al. 1982). The risk of diabetes in relatives of IDDM patients increases markedly when the number of autoantibodies detected in the serum increases (Bingley et al. 1994; Verge et al. 1994). In a group of high genetic risk, presence in serum of antibodies to one or more of these autoantigens predicted the disease onset accurately (Verge et al. 1996). Also permanently healthy subjects (as regards IDDM) may have temporarily or permanently antibodies against one of the three antigens, but antibodies against multiple antigens occur extremely rarely. It is therefore sought to simultaneously determine reactivity against two or all three of the proteins, as the positivity for more than one

of these autoantibodies remarkably increases disease risk (Bingley et al. 1994).

GAD65 (Bu et al. 1992) has several epitopes recognised by autoantibodies (Falorni et al. 1996). These are located mostly at the center and C-terminus of the molecule whereas the N-terminal quarter of the molecule is thought to contribute to membrane docking of the protein, and to contain few if any IDDM-informative epitopes (Falorni et al. 1996).

IA2 (also known as ICA512) (Rabin et al. 1994) is a transmembrane protein with still unknown function. The intracellular part of the molecule ($IA2_{ic}$, about 40 kDa) contains a domain with similarity to the active center of protein phosphatases (Fischer et al. 1991), but no enzymatic activity has been ascribed the IA2 molecule. The informative epitopes of IA2 reside in the cytoplasmic domain and herein they are concentrated at the C-terminal half (Lampasona et al. 1996; Zhang et al. 1997).

Insulin (Bell et al. 1980) is made by pancreatic β-cells as a precursor preproinsulin which is cleaved to proinsulin. The proinsulin is further processed to give the insulin consisting of A and B chains connected together with two disulphide bridges.

More than 20% of sera collected from newly-diagnosed IDDM-patients contain insulin autoantibodies (IAA) (Sabbah et al. 1996). As, however, the immunity to insulin may have arisen through formation of response to prepro- or proinsulins (Snorgaard et al. 1996), it is relevant to use these peptides in this assay system. Tolerance to this autoantigen may be induced by oral insulin feeding in non-obese diabetic (NOD) mice (Zhang et al. 1991).

In addition to linear epitopes, autoantibodies are thought to recognize important conformational epitopes resulting

from the three-dimensional structure of the protein (Kim et al. 1993). Antigen molecules produced or assayed using techniques which destroy these structures are less informative as regards IDDM or prediabetes.

Several methods for detection of autoantibodies in IDDM sera have been elaborated. One method exploits in vitro transcription-translation for producing radioactively labeled autoantigen (IA2, GAD65) (Petersen et al. 1994), while in another method biotin-labeled GAD65 is added to the patient sera and after formation of immune complexes, free label is detected and quantitated (Mehta et al. 1996). These methods all suffer from suboptimal niveau of informativity, as they employ only one specific autoantigen. Moreover they have the drawbacks associated with the use of radiochemicals.

Using a protein molecule in which a combination of the epitopes from at least two but preferably three different autoantigens are represented should detect a larger panel of autoantibodies thus revealing more specifically the population of individuals at risk of developing the disease.

SUMMARY OF THE INVENTION

According to one aspect, this invention relates to a new fusion protein having epitopes of at least two of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) and preproinsulin (PPINS) wherein said epitopes are connected with a linker peptide, said fusion protein being able to bind to a solid phase.

According to another aspect, the invention concerns a cDNA sequence encoding the said fusion protein.

According to a third aspect, the invention concerns a vector and a cell comprising said cDNA.

According to a fourth aspect, the invention concerns an immunoassay for the simultaneous determination in a sample of a person's body fluid of at least two insulin-dependent diabetes mellitus (IDDM) -related autoantibodies, wherein each autoantibody is specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS). The immunoassay comprises the steps of

- incubating said sample with said autoantigens or, alternatively, with the fusion protein according to this invention, said autoantigens or said fusion protein being bound to a solid support,
- adding at least one labeled reagent capable of binding to one or more of said autoantibodies, and
- quantifying the signals from the labels bound to the solid phase.

According to still one aspect, the invention concerns a method for diagnosing a person's risk of developing insulin-dependent diabetes mellitus (IDDM), said method comprising the determination in a sample of said person's body fluid of at least two insulin dependent diabetes mellitus (IDDM) -related autoantibodies specific for an epitope of the autoantigens glutamic acid decarboxylase (GAD65), islet cell antigen (IA2) or preproinsulin (PPINS), wherein the presence of at least two of said autoantibodies are indicative for said person's risk of developing IDDM. The order of appearance of these autoantibodies is used to predict the time point of onset of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b show the cDNA construct for a fusion protein according to this invention,

Figure 2a shows the amino acid sequence of the IA2 protein,

Figure 2b shows the amino acid sequence of the GAD65

(SEQ ID NO:6)

protein,

Figure 2c shows the amino acid sequence of preproinsulin (PPINS), (SEQ ID NO:7)

(SEQ ID NO:8)

Figures 3a-3b show the nucleotide sequence encoding GAD65,

(SEQ ID NO:9)

Figures 3c-3e show the nucleotide sequence encoding IA2,

(SEQ ID NO:10)

Figures 3f-3i show the human insulin gene,

Figure 4 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents bound to said autoantibodies, wherein the reagents are labeled with different labels, and

Figure 5 shows the fusion protein according to this invention attached to a solid support, autoantibodies attached to epitopes of said protein, and labeled reagents bound to said autoantibodies, wherein the reagents are labeled with the same label.

DETAILED DESCRIPTION OF THE INVENTION

The term "epitope" can be an amino acid sequence anything from very few (about 5 to 10) amino acids of the autoantigens up to the whole autoantigen. Preferable lengths of the epitopes are represented by the underlined amino acid sequences in Figures 2a and 2b, and the whole antigen sequence is disclosed in Figure 2c. Thus, the epitope of IA2 comprises preferably the amino acids 771-979 of the amino acid sequence shown in Figure 2a. Another preferred alternative is the whole intracellular domain (amino acids ranging from about 576 to 979 of the sequence in Figure 2a). The epitope of GAD65 comprises preferably the amino acids 102-585 of the amino acid sequence shown in Figure 2b, and the epitope of PPINS comprises preferably

all the amino acids 1-110 of the polypeptide shown in Figure 2c. It should be noted that the above mentioned specific sequences are examples only.

According to a preferred embodiment, the fusion protein has epitopes of each of the autoantigens GAD65, IA2 and PPINS. Such a fusion protein allows simultaneous detection of autoantibodies specific for any of said autoantigens.

Said fusion protein containing epitopes of GAD65, IA2 and PPINS is formed by combining these domains via short peptides consisting of amino acid residues, e.g. lysine and arginine residues.

The epitopes from distinct autoantigens will be linked together via short peptides containing e.g. several lysine residues, which allows preferential labeling of these lys-residues. For construction of the polygenic cDNA, the linker-encoding cDNA contains a recognition site for a rarely cutting restriction enzyme such as Not I or Sgf I (see Figure 1a and 1b).

These linker residues may be connected to a member of an affinity binding pair so as to enable the binding of said fusion protein to a solid phase. The bioaffinity pair may be e.g. biotin - streptavidin. The residues (lysine) can be biotinylated after which the fusion protein is attached to a streptavidin-coated solid phase. The solid phase can e.g. be a well of a microtitration strip or plate. Alternatively, the solid phase consists of microparticles.

The fusion protein can alternatively be bound to the solid phase by direct adsorption. Furthermore, the fusion protein can be covalently linked to the solid phase. In this case the fusion protein must be provided with groups able to create a covalent bond with the solid phase.
Figures 2 and 3 show the amino acid sequences and the nucleotide sequences, respectively, of the preferred

epitopes.

The following illustrates the construction of the fusion protein and its preparation.

The N-terminus of the hybrid protein will contain a flag peptide NH2-DYKDDDDK-COOH (SEQ ID NO:1) with a free N-terminal amino group to allow recognition of the protein using M1 monoclonal antibody (ATCC cell line nr. HB 9259). This enables detection of the protein in SDS-PAGE where not all monoclonals function.

At the carboxy-terminal end of the fusion protein and in the single antigens a motif X-X-G-S-H-H-H-H-H-H (SEQ ID NO:11) is introduced to allow purification of the protein with metal chelate affinity chromatography and detection with monoclonal antibody against this epitope (Cedarlane Laboratories Ltd, Canada).

The GAD65 gene (Bu et al. 1992) is, for example, amplified with PCR (nucleotides 1311-1755) in such a manner that 101 amino acid residues are removed from the N-terminus.

The 3'-end oligonucleotide contains 17 bases complementary to the mRNA of GAD65 and an additional sequence encoding half of a peptide forming the bridge between GAD65 and IA2 domains.

The nucleotide sequence of the bridge is for example

Not I (SEQ ID NO:12;)

GAD65-AAGAAGAAGCGGCCGCGAAAGAAGAAG-IA2 (amino acid sequence of the peptide KKKRPRKKK), (SEQ ID NO:2) or

Sfg I (SEQ ID NO:13;)

GAD65-AAGAAGAAGCGATCGCGAAAGAAGAAG-IA2 (amino acid sequence KKKRSRKKK) (SEQ ID NO:4). The restriction enzyme recognition sites are underlined in the middle. The fragments are made from a

plasmid harbouring said cDNAs with PCR and digested with appropriate restriction enzymes (e.g. Not I or Sfg I) and cloned into appropriate vectors. The GAD65 part is linked to IA2 and this to PPINS, using general cloning techniques.

The PPINS gene 5'-oligo contains half of the polylysine-arginine-encoding sequence with a Not I or Sfg I site for coupling to the IA2 gene 3'-end. The 3'-oligo of PPINS has a histidine hexapeptide-encoding sequence to enable antibody recognition and metal chelate chromatography purification and/or immobilization if necessary (Mauch et al. 1993).

Purified, restriction enzyme-treated PCR fragments are cloned in a FastBac derivative and E.coli DH10Bac cells are transfected with the plasmid. Recombinant clones are selected and DNA isolated and transfected into Sf9 insect cells.

Virus-producing cells are cultivated and stock virus made. Large-scale cultures are used to produce recombinant single proteins and the polyprotein.

SDS-PAGE/Western analysis is used to analyse size and immunoreactivity of the recombinant polyproteins. The proteins are blotted onto a nitrocellulose or nylon membrane and GAD/IA2/PPINS antibodies used to detect the product visualised with enhanced chemiluminescence, ECL.

For purification of the polyprotein GAD65-specific monoclonal antibody (GAD6, Developmental Studies Hybridoma Bank, Iowa University) is immobilized to Sepharose 4B activated with cyanogen bromide (Pharmacia, Uppsala, Sweden). Elution of the protein is performed at low pH (3-4) and solubility is achieved by adding detergents (e.g. Nonidet or Tween) to allow dissociation from the membranes.

The steps from cloning to large scale production can be

described in more detail as follows:

1. Cloning into the pK503-9 vector (Kari Keinänen VTT Finland), a derivative of pFastBac (Gibco BRL Paisley Scotland) of GAD65, or IA2 or PPINS gene, each containing a flag recognition signal (FLAG^R, Immunex Corporation) for antibody detection and a signal peptide for ecdysone glucotransferase (EGT) for transport into the endoplasmatic reticulum for removal of the signal peptide with simultaneous release of N-terminal aspartate for M1 antibody recognition. The constructs contain each a X-X-G-S-H-H-H-H-H-H (SEQ ID NO:11) carboxyterminal peptide to allow metal chelate affinity purification and detection with specific antibody (Cedarlane, Canada) of the product.

2. Transformation into competent E. coli DH10Bac cells of the plasmids containing the single genes.

3. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.

4. Production of recombinant stock virus.

5. Large scale production of the proteins.

6. Cloning into pK503-9 vector of a cDNA construct for the fusion protein (FP) comprising GAD65 (nt 1311-1755; aa 102-585)-IA2(nt 2313-2937; aa 771-979)-PPINS (nt 2424-2610 and 3396-3539 (of the genomic DNA sequence, accession No. V00565); aa 1-110) in all alternative orders.

7. Transformation into competent E. coli DH10Bac cells of the plasmids containing the fusion protein.

8. Isolation of recombinant Bacmid DNA and transfection with the fused DNA of the Sf9 or Hi-5 insect cells.

9. Production of recombinant stock virus.

10. Large scale production of the fusion protein.

In case the baculovirus expression system does not work optimally, alternative systems such as E.coli, yeast, or in vitro transcription translation assay (Petersen et al. 1994) will be used for production of said polypeptides.

The present invention relates further to the use of the fusion protein in an immunoassay for the detection of several pancreatic beta-cell autoantibodies in IDDM patients and prediabetic sera. The assay may detect patients at risk of developing IDDM, i.e. having a pre-IDDM condition. As a multicomponent assay, the method could also be used to predict the time point of onset of the disease. The methodology which combines epitopes of several islet beta cell autoantigens increases the informativity and prediction value of the test aimed at prediction of risk and onset of disease in individuals genetically predisposed to IDDM.

In the immunoassay according to this invention, a sample of the person's body fluid (e.g. serum) is incubated with the fusion protein bound to a solid surface, e.g. a microtitration plate. The bound autoantigens are thereafter detected with a labeled reagent. The reagents can be the single autoantigens GAD65, IA2 and PPINS; or proteins comprising epitopes thereof. These reagents are used to detect free antigen-binding regions (V-regions) on the bound autoantibodies. One variant of the method will be used for differential detection of the individual autoantigen specificities of the antibody in one assay if individual autoantigens (AAGs) labeled with three different labels are used (see Figure 4). Alternatively, when the polyprotein (the fusion protein) is labeled with only one label, it can be used to reveal the sum of these three reactivities in the sample (Figure 5). The same result is achieved if the single antigens are all labeled with the same label. The labeled reagent can further be an anti-

human monoclonal antibody. In this case the assay can reveal only the sum of the three autoantibodies.

The technique which involves use of the label attached to the fusion protein or individual autoantigens circumvents several problems encountered in the conventional assays. First, there is little or no nonspecific binding to the vials due to the fact that the carrier surfaces have already been blocked with the corresponding antigen. Second, the attachment via a bioaffinity pair such as streptavidin/biotin interaction to the vial and use of a flexible peptide between the individual antigenic epitopes enable free motion and folding of the protein in the solution (Figure 5).

The label can be any suitable label. However, according to a preferred embodiment, the label is a lanthanide. In case three different labels are used, said labels can be e.g. Eu, Sm, Tb and Dy (Siitari et al. 1990; Hemmilä et al. 1993). In such a case the detection is based on time-resolved fluorescence.

The free labeled reagent can be removed after the incubation step before the signal is quantified (heterogeneous assay), or the signal can be quantified without foregoing removal of the free labelled reagent (homogeneous assay).

The procedures are preferably automatized. Automatization of the procedures involves laboratory robots which apply samples onto cover slips and the fluorescence is detected in an micro array system in an appropriate unit (Wallac OY, Finland).

The simultaneous detection of antibodies against the three autoantigens increases the capacity to process large sample series. The use of a micro array system substantially increases the capacity. This has become necessary as

nationwide screenings of newborns are undertaken in several research centers.

The test principle using time-resolved fluoroimmunoassay (TR-FIA) offers an extremely sensitive means for detection of autoantibodies with minimum amount of nonspecific reactivity due to used specific antigen label. The longevity of the lanthanide label is also an advantage as compared to radiolabel. LA Better than radiolabel

The system allows retaining of important conformational epitopes of the antigen as immobilization of the polyprotein is via specific flexible intervening sequences and causes minimal tortion to the antigen.

The following illustrates the use of the fusion protein in an immunoassay:

To the polyprotein (fusion protein) biotin is bound in limiting conditions to prevent other than the lysine residues of the linker peptide to be biotinylated. Streptavidine-coated microscope slides are treated with biotin - fusion protein and the residual sites are blocked with bovine serum albumin or another suitable binding protein.

M1 flag-specific monoclonal antibody will be used to monitor binding onto solid support of free recombinant autoantigens while autoantigen-specific monoclonals (e.g. GAD1, GAD6, MICA-3 (Boehringer) etc.) will be used to detect availability of specific epitopes. After incubation with sample sera, Eu-labeled GAD65, Sm-labeled IA2 and Tb-labeled PPINS (produced as a single protein with the baculosystem) are printed robotically onto the microscope slides in four quadrants covering an area of about 1 cm^2, allowed to bind, washed and dried in vacuum, and the fluorescence is measured on TR fluorometer.

The functionality of the method is tested using IDDM sera known to be positive for one or more of the antigens used.

For specificity testing recombinant GAD65, IA2 and PPINS, or fusion protein are added into patient sample to preadsorb specific antibodies.

The informativity will be compared with conventional systems. Statistical tests will be used to create best possible segregation of the positive and negative assay values.

The high density array system is fully automatized.

The invention is further illustrated by the following examples.

Example 1

Labeling procedure

Isothiocyantophenyl-DTTA-Eu, or Tb, or Sm (Mukkala 1989) will be used for labeling of the FP or the single autoantigens. Mainly the protocols of Lövgren & Petterson (1990) and Hemmilä et al. (1984) will be followed. 30-100 fold molar excess of the label substance will be used giving approximately 10-12 lanthanide molecules per protein molecule. For Tb, 500 fold excess will be used. The coupling is carried out for 18 hr at 0 °C in 0.1 M bicarbonate buffer pH 9.2. The Eu (Tb,Sm)-AAg complex is separated from free Eu (Tb, Sm) by gel filtration in a Sepharose 6B column equilibrated with 0.05 M Tris-HCl buffer pH 7.75 containing 0.9% NaCl and 0.05% NaN_3. The Eu-AAg complex is stored at 4 °C.

Example 2

Immunoassay

The assay is performed in the wells of polystyrene microtitration strip coated with unlabeled autoantigen preparate for 18 hr at 25 °C in 0.1 M bicarbonate buffer pH 9.6 (Siitari & Kurppa 1987). The strips are washed prior to use with 0.9% NaCl containing 0.05 % Tween 20 and 0.3% Germal II. To each well 100 µl of diluted (1:10) serum is added and incubated for 1 hr at 40 °C, washed 2x with the wash solution and 200 µl of the Eu-labeled autoantigen fraction (50 ng/well) is added.

The strips are incubated for 1 hr at 40 °C. The strips are washed 5x with the washing solution. Thereafter Enhancement Solution (EG&G Wallac) 200 µl/well is added. Strips are shaken for 10 min in a plate shaker and measured in EG&G Wallac Victor fluorometer for 1s/specimen. The photons emitted are measured as counts/s. Automated data reduction program calculates mean value of duplicates and the coefficient of variation (CV%).

For future development, the assay formate will be miniaturized e.g. by immobilizing the autoantigen molecules onto microparticles (Lövgren et al. 1997) or as a microarray onto glass cover slips.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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